camelpox virus (CMLV) and cowpox virus (CPV) have raised questions about potential differences in VACV, CPV and CMLV replication cycles and the possible presence of other targets. Based on these data, we investigated the relative quantity of enveloped virus versus non-enveloped virus in the medium and cell lysates using density gradients performed on [methyl-^{3}H]-thymidine labeled VACV, CPV and CMLV infected cells, either in the absence or presence of ST-246. We then characterized ST-246 resistant orthopoxviruses which were selected following increasing concentrations of ST-246 for approximately 17 passages. Analysis of cesium chloride gradient fractions indicated that most of the viruses produced during a productive VACV, CPV and CMLV infection were enveloped. The spread of VACV appeared to involve both intra- and extracellular enveloped forms. In contrast, CPV and CMLV produced few extracellular enveloped forms and seemed to propagate via intracellular infectious particles. These data indicated a difference in the way of propagation of CMLV/CPV and VACV. It was also clear that the antiviral activity of ST-246 was due to an interference with the formation of enveloped forms and that the discrepancy in the inhibitory effect of ST-246 on orthopoxvirus replication was more likely due to differences in orthopoxvirus spread. The IC_{50}s determined for each ST-246 resistant virus mutant were at least 100–2000-fold higher than those of the wild-type viruses. Sequencing of the F13L genes of both resistant and wild-type VACV, CPV and CMLV strains is currently ongoing. We have demonstrated the antiviral potency of ST-246 on the production of enveloped orthopoxvirus particles and confirmed differences in VACV, CPV and CMLV replication cycles. ST-246 resistant viruses have been plaque purified and the determination of mutations induced by ST-246 in the F13L gene is currently under investigation.

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Vaccinia DNA Polymerase is Profoundly Inhibited by Cidofovir and (*S*)-HPMPA Incorporated into the Template Strand

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Cidofovir (CDV) and (*S*)-9-[3-hydroxy-(2-phosphonomethoxy)propyl]adenine (HPMPA) are analogs of dCMP and dAMP, respectively, and are effective inhibitors of poxvirus replication. However, the precise mechanism by which these drugs inhibit viral growth remains unclear. We have previously used vaccinia virus as a model system to show that the diphosphoryl metabolites of these drugs are substrates for vaccinia DNA polymerase, but once incorporated into the penultimate 3′-end of the primer strand inhibit both the polymerase and 3′-to-5′ exonuclease activities. Interestingly, although HPMPA exhibits a much lower EC_{50} than CDV, HPMPApp is not nearly as effective an inhibitor of primer extension and exonuclease activity in vitro compared to CDVpp. This suggests that other mechanism(s) must account for the differences in relative activities of the two drugs. To investigate these mechanisms, we examined the effects of CDV and HPMPA on vaccinia DNA polymerase activity when incorporated into the template strand. These templates were prepared using a two-step enzymatic method. First, oligonucleotide-primed templates were prepared containing a single dGMP or dTMP residue (to direct CDV or HPMPA incorporation, respectively) and multiple dUMP residues. Primer extension was used to incorporate each drug, and then the dUMP-containing strand was degraded using uracil DNA glycosylase. Labeled primers were then annealed to the newly extended strands and we tested whether vaccinia DNA polymerase could extend these primers back across the drug residue. We found that although the correct nucleotide could be incorporated opposite the drug lesion, further extension by the DNA polymerase was blocked. Control templates, containing either dCMP or dAMP at the same sites, did not show this block. These results suggest that although primer extension is slowed by the incorporation of these drugs, the profound effects on DNA replication are more likely caused by drug incorporated into the template strand. HPMPApp is a much better substrate for vaccinia DNA polymerase than is CDVpp, but this makes it the more effective drug because more HPMPA is then incorporated into the template strand.

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Development of a Model for the Study of Antivirals Against Molluscum Contagiosum Virus (MCV)

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Molluscum contagiosum virus (MCV) and variola virus (VARV) are the only two poxviruses that are specific for humans. VARV has been eradicated from the human population through extensive programs of vaccination. MCV is present worldwide and is directly passed by direct skin contact to produce cutaneous and, rarely, mucosal lesions. It occurs predominantly in preadolescent children, sexually active adults, and in individuals with impaired immunity. The study of MCV has been hampered by the lack of an in vitro system that allows virus replication. We describe here the growth of MCV in 2D (two dimensional) and 3D cultures of primary human keratinocytes (PHKs). PHKs were isolated from neonatal foreskins and used to prepare monolayer cultures and organotypic raft cultures, which are able to mimic fully differentiated skin. Fresh lesions obtained from preadolescent children were used to recover the MCV. Organotypic epithelial raft cultures were infected with different MCV fresh isolates and after a period of approximately 25 days were processed for histological examination. A typical cytopathic effect characterized by the appearance of huge infected cells, with internal organelles dislocated and obliterated by a large intracytoplasmic

inclusion (Henderson-Patterson inclusion bodies or Molluscum bodies) was observed. When raft cultures were incubated for a shorter period of time, few infected cells were predominantly observed in the upper layers of the raft cultures. Several clinical isolates of MCV have been successfully passaged in monolayer cultures of PHKs. Furthermore, the presence of MCV could be confirmed following electron microscopy analysis both in 2D and 3D cultures. Cells contained the characteristic mature, intracellular brick-shaped virions associated with other poxvirus infections. In conclusion, MCV isolates induced a characteristic cytopathic effect on normal human keratinocytes grown as monolayer or in a differentiated epithelium allowing the study of MCV replication and development of antivirals.

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In Vitro and *In Vivo* Efficacy of a Pyrimidine Nucleoside Analog Against Vaccinia and Cowpox Viruses

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A novel nucleoside analog, SRI 21950, a 4′-thionucleoside, was synthesized and evaluated for activity against vaccinia (VV) and cowpox viruses (CV). In cell culture, SRI 21950 was effective at concentrations less than 1.0 μM against wild-type VV and CV. In addition, it retained its antiviral activity against cidofovir-resistant and ST-246-resistant strains. A thymidine kinase negative strain of VV exhibited reduced susceptibility to the drug suggesting that it may be specifically activated by this enzyme. In vitro cytotoxicity was measured by neutral red uptake and CellTiter-Glo® cell viability assays and indicated a cell cytotoxic (CC_{50}) value of greater than 100 μM for this compound using either method. To determine if this compound had activity in vivo, mice were lethally infected intranasally with either VV or CV. In the initial experiments, SRI 21950 was administered i.p. twice daily at 5, 15 or 50 mg/kg beginning 24 h post-VV infection and continued for 5 days. Treatment with SRI 21950 completely protected VV-infected mice from mortality at all doses ($P < 0.001$). In a second experiment, SRI 21950 was administered i.p. twice daily at 1.5, 5 or 15 mg/kg beginning 24 h after infection with CV and continued for 5 days and again treatment resulted in complete protection from mortality at all doses ($P < 0.001$). To determine if SRI 21950 had activity when administered orally, the compound was given by oral gavage twice daily at 5, 15 and 50 mg/kg. Again, a significant reduction in mortality at all doses ($P < 0.001$) was observed. Additional studies using lower doses of SRI 21950 initiated several days after CV or VV infection are in progress to help determine the potential of this compound, however, the results to date indicate that SRI 21950 has promise for treatment of adverse reactions to smallpox vaccinations, monkeypox or smallpox disease.

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Effect of Treatment with the Cidofovir Analogue HDP-CDV in Guinea Pig Models of Cytomegalovirus Infection

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Congenital cytomegalovirus (CMV) infection can be life-threatening and often results in significant sequelae including neurosensorial hearing loss. Currently available anti-CMV antivirals are effective, but their use is limited due to the lack of an effective oral formulation and the significant side effects associated with therapy. We evaluated hexadecyloxypropyl-CDV (HDP-CDV), an orally active ether lipid ester analogue of cidofovir in guinea pig models of congenital CMV infection. Pregnant Hartley guinea pigs were inoculated SQ with 1×10^5 pfu GPCMV during the late second/early third trimester of gestation. HDP-CDV (20 mg/kg, $N = 5$) or placebo ($N = 4$) was administered PO at 24 h post-infection (p.i.) and 7 days p.i. to pregnant animals. Pup survival was increased in the drug treated group (15/16, 93.8%) compared to placebo (10/18, 62.5%, $P = 0.02$). The viral load, examined by real-time PCR, in tissues harvested from pups sacrificed within 7 days of birth was significantly ($P < 0.05$) lower in the spleen (1.7 ± 0.7 $\log_{10}$ copies/μg DNA) and the liver (1.9 ± 0.8 $\log_{10}$ copies/μg DNA) of drug treated pups compared to the controls (2.5 ± 1.1 $\log_{10}$ copies/μg DNA and 2.9 ± 1.5 for the spleen and liver, respectively). Further evaluation of treatment on viral replication was performed in a guinea pig model in which viable newborn strain 2 guinea pigs were inoculated IP with GPCMV (1×10^6 pfu) within 48 h of life. Pups then received either HDP-CDV (4.0 mg/kg PO, $N = 12$) or placebo ($N = 11$), beginning 24 h p.i. and continued for 10 days. All pups were sacrificed on day 10 p.i. and the tissues were harvested for evaluation of the viral load by real-time PCR. The viral load in the spleen, liver, lung and brain of drug treated animals was significantly ($P < 0.005$) lower (1.3–2.8 $\log_{10}$) when compared to controls. These results indicate that oral HDP-CDV is well tolerated and effective in limiting CMV infection in these models and may provide an oral alternative to other therapies.

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